JOURNAL OF BACTERIOLOGY, Dec. 1988, p. 5654–5661
0021-9193/88/125654-08$02.00/0

Vol. 170, No. 12

Role of the Leader Peptide of Maltose-Binding Protein in Two Steps of the Export Process

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During the process of export of maltose-binding protein to the periplasm of *Escherichia coli*, the leader peptide is involved in at least two steps. The presence of the leader portion of maltose-binding protein was shown to be necessary to mediate initial binding of the precursor to the membrane. However, the presence of a mutationally altered leader which does not sustain export in vivo was sufficient to allow this interaction. Thus, the defect in export which is manifested in vivo by this mutational substitution occurs at a step that follows membrane association, most likely the translocation step. Translocation occurs at discrete sites that are not uniformly distributed over the cytoplasmic membrane. A large proportion of the membrane involved in translocation has a higher density than that of bulk cytoplasmic membrane.

Newly synthesized polypeptides are specifically translocated across membranes during secretion and biogenesis of organelles in eucaryotes and during subcellular compartmentalization of proteins in procaryotes. The different systems appear to represent variations on a common theme. Each involves synthesis of proteins as precursors that have amino acid extensions which are proteolytically removed during or immediately following traversal of a membrane (1, 28, 37). In addition, there is evidence that in each system polypeptides are competent for transfer through membranes only when they are devoid of the stable tertiary structure characteristic of the corresponding mature species (11, 12, 27, 33, 40).

Maltose-binding protein is exported by the gram-negative bacterium *Escherichia coli* from the cytosol to the periplasm, an aqueous compartment that lies between the cytoplasmic membrane and the outer membrane. In the work described here, the role of the leader sequence during two steps of the export process was analyzed. It was demonstrated that the presence of a leader sequence was necessary to mediate entry into the export pathway; however, even the presence of an altered leader that did not sustain export in vivo was sufficient to allow the initial interaction with the membrane. The defect was expressed during the subsequent translocation step. The cellular membranes were separated by density, and operational definitions of an entry site and a translocation site were established: initially, precursor maltose-binding protein associates with membrane that has the density characteristic of bulk cytoplasmic membrane, and subsequent translocation occurs at discrete sites that have a higher density.

MATERIALS AND METHODS

Materials. [^{35}S]methionine (approximately 1,000 Ci/mmol) was purchased from New England Nuclear Corp., and protein A-Sepharose CL-4B was obtained from Pharmacia Fine Chemicals. Lysozyme, protease type XI (proteinase K), chloramphenicol, phenylmethylsulfonyl fluoride, sucrose (grade I), and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) were from Sigma Chemical Co. Amplify was purchased from Amersham, and metrizamide was from Nycomed AS Diagnostics. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was a gift from Dupont.

Bacterial strains and growth conditions. Strains used were *E. coli* K-12 strain G6 (Hfr *his*) or *E. coli* K-12 strain MC4100 (F$^-$ *lacU169 araD139 rpsL150 thi flbB5301 ptsF25 relA1*) (7) and its isogenic derivatives MC4100 carrying *malE18-1* (13) and MM18 (3). Strain JP27, constructed by P. Bassford and John Puziss, is strain BAR1091 (31) (which itself is derived from MC4100) harboring the plasmid pUZ226, which carries *malEΔ2-26* under the *lacUV5* promoter-operator control region. The MalE polypeptide produced has no leader sequence; the initiating methionine is followed immediately by the first amino acid of the mature protein. The first 23 amino acyl residues were identified by using an Applied Biosystems 470A protein sequencer. Strains were grown at 35°C in M9 minimal salts medium (20) supplemented with 0.4% glycerol and 0.2% maltose except where otherwise indicated. When the strain used was G6, histidine (0.01%) was added to the minimal salts medium. Strain JP27 was grown without addition of the inducer isopropylthiogalactoside (IPTG) to ensure that the maltose-binding protein would not saturate a cellular component because it was produced in quantities exceeding the normal level. The amount of maltose-binding protein produced in the conditions used was approximately 10 to 20% of the amount produced from the fully induced normal chromosomal copy of *malE*. To be sure that the protein analyzed was being produced uniformly in the culture at a low level and not overproduced by a minor proportion of the cells, the inducer IPTG was added to a sample of the culture after labeling, and it was shown that the culture retained the capacity for overproduction of maltose-binding protein.

Labeling with [^{35}S]methionine. When exponentially growing cultures reached a density of 4×10^8 cells per ml, [^{35}S]methionine was added at between 6 and 60 μCi/ml (specific activity, 52 to 670 Ci/mmol). The length of the labeling period and the procedure for termination of labeling varied among experiments. During all manipulations following termination of labeling, precautions were taken to avoid warming samples to temperatures above 4°C. Cells were harvested by centrifugation for 1 min at 10,000 rpm with the SS-34 rotor in a Sorval RC-5B centrifuge.

Labeling conditions. Labeling conditions used for the experiments shown in Fig. 4 were as follows.

(i) Distribution of wild-type precursor accumulated by

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dissipation of proton motive force. A culture of exponentially growing *E. coli* K-12 strain G6 producing wild-type maltose-binding protein was treated with CCCP (50 μM) for 1 min before addition of [^{35}S]methionine. After addition of the radioisotope, incubation of the culture was continued for 1 min. Chloramphenicol was added to 100 μg/ml, and the culture was immediately transferred to an ice-water bath and swirled for 1 min.

(ii) Distribution of wild-type maltose-binding protein accumulated after inhibition of protein synthesis. A culture of exponentially growing *E. coli* K-12 strain G6 producing wild-type maltose-binding protein was labeled for 15 s with [^{35}S]methionine. Incorporation of radioisotope was terminated by the addition of chloramphenicol (100 μg/ml), and the culture was immediately transferred to an ice-water bath and swirled for 1 min.

(iii) Distribution of mutated precursor that carries a defective leader peptide. A culture of exponentially growing *E. coli* K-12 strain MC4100 carrying the mutation *malE18-1* was labeled for 15 s with [^{35}S]methionine. Incorporation of the radioisotope was terminated by the addition of chloramphenicol (100 μg/ml), and the culture was immediately transferred to an ice-water bath and swirled for 1 min.

Preparation of cellular lysates and gradient centrifugation. (i) Flotation gradient centrifugation. When lysates were to be analyzed by flotation gradient centrifugation, cells were first converted to spheroplasts by the method of Witholt et al. (38) to release periplasmic proteins. The cell density during spheroplast formation was 1.5×10^9 cells per ml. Spheroplasts were stabilized by addition of $MgSO_4$ to a final concentration of 10 mM and subjected to centrifugation for 40 s in an Eppendorf centrifuge (model 5414) to separate them from periplasmic proteins. The spheroplasts were suspended in 10 mM HEPES (pH 7.6)–5 mM EDTA and disrupted by five pulses (15 s each) of sonication in a cuphorn sonicator (Tekmar) filled with an ice-water mixture. The lysed spheroplasts were subjected to flotation gradient centrifugation. A sample containing material from between 2×10^8 and 4×10^8 cells was brought to a density of 1.29 g/ml with metrizamide. Sixty-five microliters of the sample was applied to the bottom of a centrifuge tube for the TLA100.1 rotor (for use in a Beckman TL100 ultracentrifuge). A solution (0.435 ml) of metrizamide in 10 mM HEPES, pH 7.6, of density 1.27 g/ml (0.435 ml) was layered over the sample. The gradients were centrifuged for 5 h at 100,000 rpm with acceleration and deceleration settings of 9. After centrifugation, six fractions of 83 μl each were withdrawn successively from the top of the gradient, and the index of refraction of each fraction was determined to allow calculation of the density. Metrizamide was used in these gradients instead of sucrose because the low viscosity of metrizamide allows membranes to reach their buoyant density more rapidly than they would in a comparable gradient of sucrose.

(ii) Sedimentation gradient centrifugation. The cells were suspended to a final density of 2×10^{10}/ml in 10 mM HEPES (pH 7.5)–5 mM EDTA, and lysozyme was added to a final concentration of 0.1 mg/ml. The cell suspensions were held in an NaCl-ice-water bath and disrupted by three pulses of sonication of 5 s each at 30-s intervals in a Tekmar sonic disruptor. For each fractionation described, the sample contained material from 2×10^{10} cells in a volume of 1 ml. The sample was applied to the top of a sucrose density gradient formed by layering sucrose solutions (buffered with 10 mM HEPES [pH 7.5]) into a centrifuge tube as follows: 56% sucrose, 2 ml; 42% sucrose, 4.75 ml; 25% sucrose, 4.25 ml. Separation of components took place during centrifugation at 170,000 × *g* (Beckman SW41 rotor) for 22.5 h. After centrifugation, fractions (1 ml) were collected and the A_{280} was monitored with an Instrumentation Specialties Company gradient fractionator.

The density of sucrose in each fraction was determined from the index of refraction. One percent of each fraction (material from the equivalent of 2×10^8 cells) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to display the protein composition. Ten percent of each fraction (corresponding to 2×10^9 cells) was used to determine the activity of β-NADH oxidase (22). For determination of the distribution of 2-keto-3-deoxyoctonate (KDO) across the gradient, 0.15 ml of each fraction, containing material from 3×10^9 cells, was brought to a volume of 1 ml in 10% trichloroacetic acid. The acid-precipitable material was collected by centrifugation (15 min, Eppendorf centrifuge). The supernatant was discarded, and in order to remove residual sucrose, the pellet was washed twice with water (1 ml) without suspension of the pellet between centrifugations. The amount of KDO in the samples was determined by the thiobarbituric acid method (24) with the following modifications: H_5IO_6 was used in place of HIO_4, and cyclohexanone was used in place of butanol with 5% HCl.

Immunoprecipitation of polypeptides related to maltose-binding protein. Samples were made to be 0.5% sodium dodecyl sulfate and 10 mM Tris hydrochloride, pH 7.8. After incubation at 55°C for 10 min, 1 ml of 1% Triton X-100–10 mM Tris hydrochloride, pH 7.8, was added to each sample. The samples were subjected to immunoprecipitation with antiserum specific for maltose-binding protein by one of two techniques. For direct immunoprecipitation, 7 μl of antiserum was added to the solubilized samples, and the samples were processed as described before (25). Alternatively, immunoprecipitation was carried out with protein A. The antibody was bound to protein A-Sepharose CL-4B before it was added to the samples (27). The immunoprecipitates were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (26), followed by autoradiography.

Assessment of conformation via sensitivity to degradation by proteinase K. The assay of conformation of precursor maltose-binding protein has been described in detail elsewhere (27). It is based on the observations that precursor maltose-binding protein that is devoid of stable structure is completely degraded by protease, whereas stably folded precursor has a structure similar to that of mature protein and thus is resistant to proteolytic degradation. Treatment of folded precursor with proteinase K results in removal of the leader and recovery of the remainder of the protein in a form that migrates at the position of mature protein during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Determination of the conformational state of wild-type precursor was carried out with strain MM18, a derivative of MC4100 that carries a *malE-lacZ* gene fusion on the λ transducing phage λp72-47 in addition to the normal *malE* gene. The culture was grown in minimal medium with 0.4% glycerol as the carbon source. When the culture had reached a density of 3×10^8 cells per ml, maltose was added (final concentration, 0.2%) to induce expression of the *malE-lacZ* gene. The product of the fused gene, a chimeric protein comprising the amino terminus of maltose-binding protein fused to β-galactosidase, cannot be exported but interacts with the export apparatus in a way that results in accumulation of precursors of exported proteins, including the precursor of maltose-binding protein (15). Thirty minutes after addition of maltose, the culture was labeled for 15 s with [^{35}S]methionine.

The uncoupler CCCP was added, the cells were harvested, and the lysate was prepared and subjected to sedimentation gradient centrifugation as described above. The identification of precursor in the various fractions as membrane associated was confirmed by flotation centrifugation. Determination of the conformational state of the mutated precursor MalE18-1 was carried out with fractions from a gradient similar to that shown in Fig. 4D. The gradient fractions to be tested were incubated under the conditions described in the text and then transferred to ice. A portion of each fraction was then incubated with proteinase K at a final concentration of 25 μg/ml, and an equal portion served as a control. After incubation on ice for 20 min, the protease inhibitor phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. After 2 min on ice in the presence of the protease inhibitor, the suspensions were brought to 5% trichloroacetic acid. The acid-precipitated samples were then processed for immunoprecipitation and analyzed by sodium dodecyl sulfate–11% polyacrylamide gel electrophoresis and autoradiography. The amount of protein in bands on autoradiograms was quantified with a Helena Laboratories Quick Scan densitometer.

RESULTS

Cellular location of precursor synthesized with and without a leader sequence. In order to define the role of the leader sequence in each of the stages of export, we compared the subcellular location of three species of maltose-binding protein: the first carries the wild-type leader, the second (MalE18-1) carries a mutated leader that does not support export in vivo (2, 5), and the third species (MalEΔ2-26) has no leader sequence. The posttranslational translocation of wild-type maltose-binding protein is a rapid process, having a half-time of 4 s at 37°C (16). Therefore, to detect newly synthesized protein in the process of being exported, it was necessary to label the cells with [^{35}S]methionine for very short times and to stop cellular processes as rapidly as possible. Fractionation studies gave essentially the same results whether the cultures were rapidly frozen 15 s after addition of the [^{35}S]methionine or were treated with the uncoupler CCCP 8 s after the addition of the [^{35}S]methionine and then transferred to ice after a total incubation time with the isotope of 15 s. The cells were harvested and converted to spheroplasts to remove the periplasm, which contains the matured maltose-binding protein. The spheroplasts were lysed, and the subcellular distribution of the various species of maltose-binding protein was determined. The technique used for analysis was flotation centrifugation; the samples were brought to a density of 1.29 g/ml with metrizamide and placed at the bottom of centrifuge tubes. A solution of metrizamide having a density of 1.27 g/ml was layered over each sample. During centrifugation, the metrizamide sedimented to establish a gradient of density, and cellular proteins were distributed within the gradient as shown in Fig. 1. Cytosolic, proteinaceous components remained near the bottom (fractions 5 and 6; density, 1.29 to 1.45 g/ml), whereas proteins associated with membrane floated to the top of the gradient where the density (fractions 1 and 2; 1.12 to 1.25 g/ml) corresponded to the buoyant density of the membranes. The distribution within each gradient of newly synthesized maltose-binding protein was quantified by immunoprecipitation in combination with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The species of maltose-binding protein that was synthesized without a leader sequence remained at the bottom of the gradient, whereas the majority of both precursor species, that carrying the wild-type leader and that carrying the defective leader, were membrane associated (Fig. 2). Discrete species of nascent polypeptides in the process of elongation accumulate transiently during the synthesis of maltose-binding protein (29) and can be readily identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see Fig. 4, nMBP). These discrete nascent species exhibited the same distribution in the flotation gradient as did the corresponding fully elongated species (Fig. 2). When the polypeptides were synthesized with a leader initially present, whether the wild-type or the mutant leader, they were membrane bound; whereas if the sequence encoding the leader had been deleted from the gene, the nascent species were free in the cytoplasm (i.e., did not float). We conclude that the presence of a leader peptide is necessary for association with the membrane; however, the presence of a leader that has a substitution of arginine for methionine in the hydrophobic core fulfills this function. Thus, the defect in export that is manifested in vivo by this mutational substitution must occur at a stage that follows membrane association. Subsequent steps that could be affected by the

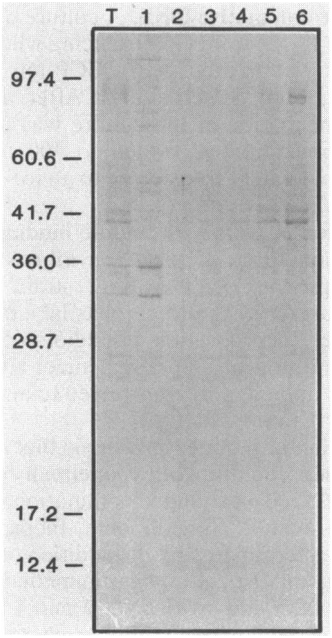

FIG. 1. Pattern of cellular proteins present at steady state separated by flotation gradient centrifugation. A culture of exponentially growing *E. coli* K-12 strain MC4100 was labeled with [^{35}S]methionine. The uncoupler CCCP (50 μM) was added 8 s after addition of the isotope. After an additional 7 s, chloramphenicol (100 μg/ml) was added, and the culture was immediately transferred to an ice-water bath and swirled for 1 min. Cells were prepared for analysis by flotation gradient centrifugation as described in Materials and Methods. After fractionation of the gradient, a volume of each fraction containing the equivalent of material from 2×10^7 cells was analyzed by electrophoresis on a sodium dodecyl sulfate–14% polyacrylamide gel. The pattern shown is stained with Coomassie brilliant blue. Lane T represents 1/3 of the amount of lysate applied to the gradient, and lanes 1 through 6 represent fractions 1 through 6. The density of metrizamide in the gradient increases from 1.16 g/ml in fraction 1 to 1.40 g/ml in fraction 6. The standards for molecular weight were: phosphorylase B (M_r 97,400), α-amylase (M_r 60,600), actin (M_r 41,700), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 28,700), myoglobin (M_r 17,200), and cytochrome *c* (M_r 12,400).

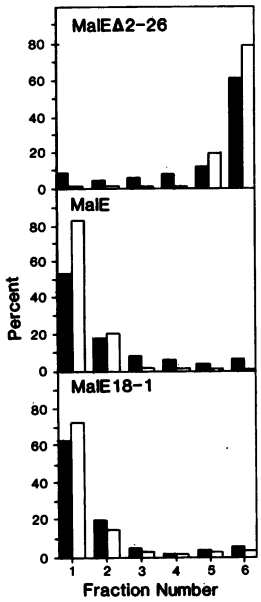

FIG. 2. Subcellular distribution of nascent and precursor maltose-binding protein. Cells were labeled and prepared for analysis by flotation gradient centrifugation as described in the legend to Fig. 1. A volume of each fraction from the flotation gradients (similar to that shown in Fig. 1) containing the equivalent of material from 2 × 10^8 cells was analyzed by immunoprecipitation with protein A, followed by electrophoresis on a sodium dodecyl sulfate–11% polyacrylamide gel and fluorography. The amount of precursor and nascent species of maltose-binding protein in each fraction was determined by densitometric tracing of bands on the fluorogram. Solid bars represent the fully elongated form of maltose-binding protein made in each of the three strains shown. Open bars represent a discrete nascent species of maltose-binding protein (see Fig. 4, nMBP.).

mutational substitution would include transfer from an entry site on the membrane to a translocation site if such sites were physically separate, transfer of the polypeptide through the membrane, and release to the periplasm.

Operational definition of subclasses of membrane involved in initial interactions and in translocation. In an effort to define the mutational block more precisely, we resolved the membranes of the envelope by sedimentation centrifugation through a gradient of sucrose and compared the distribution of wild-type precursor with that of mutant precursor. The cytoplasmic membrane and outer membrane were well separated, as indicated by the protein composition (Fig. 3B) of each fraction and by the distribution of β-NADH oxidase activity, a marker for cytoplasmic membrane, and of KDO, a component of lipopolysaccharide, which is exclusively located in the outer membrane (Fig. 3A). This fractionation procedure yields a highly reproducible pattern not only for the distribution of cellular components that are present at steady state, but also for the distribution of species that are intermediates in the pathway of export of maltose-binding protein. With variations in the labeling conditions, this fractionation was carried out more than 50 times, and the results obtained are in agreement with those presented here. The patterns of distribution of intermediates in strains producing wild-type maltose-binding protein were essentially identical whether the strain used was *E. coli* K-12 G6 or *E. coli* K-12 MC4100. Immediately after the 15-s labeling period, half of the newly synthesized mature protein was found

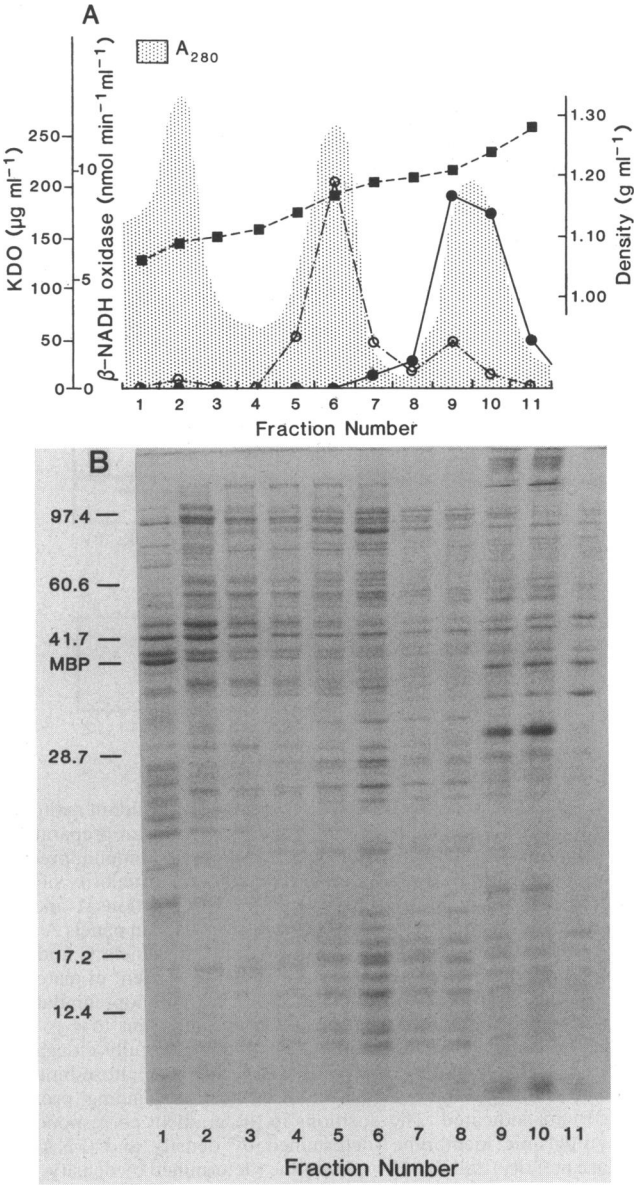

FIG. 3. Fractionation of cellular lysates by sedimentation gradient centrifugation. An exponentially growing culture of *E. coli* K-12 strain G6 producing wild-type maltose-binding protein was labeled with [^{35}S]methionine for 15 s. Labeling was terminated by pouring the culture (50 ml) into a Pyrex crystallization dish (170 by 90 mm) held in a bath of dry ice-ethanol. The culture was thawed while on ice. Preparation of the cellular lysate and sedimentation gradient centrifugation were done as described in Materials and Methods. (A) Position of cytoplasmic membrane and outer membrane. The position of cytoplasmic membrane is indicated by the activity of β-NADH oxidase (○) and the position of outer membrane by the presence of KDO (●). The relative absorbance at 280 nm (A_{280}) is shown by the stippled area. The density of sucrose in each fraction is indicated (■). (B) Pattern of cellular protein separated by sedimentation gradient centrifugation. A volume of each fraction containing the equivalent of material from 2 × 10^8 cells was analyzed by electrophoresis on a sodium dodecyl sulfate–15% polyacrylamide gel. The pattern shown is stained with Coomassie brilliant blue and thus represents the level of proteins at steady state. The position of mature maltose-binding protein, M_r 38,500, is indicated (MBP). The standards for molecular weights are as in Fig. 1.

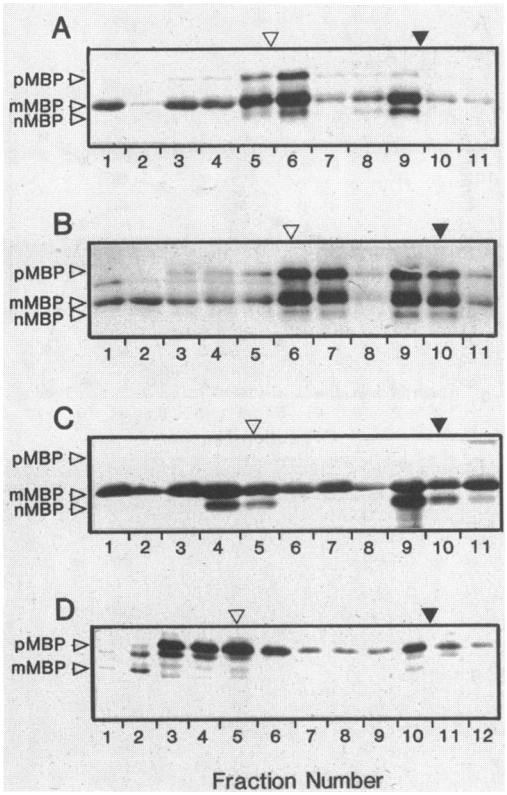

FIG. 4. Subcellular distribution of wild-type and mutant maltose-binding protein under various conditions. For details of preparation of cellular lysates, sedimentation centrifugation, and immunoprecipitation, see Materials and Methods. Since the great majority of the matured maltose-binding protein was found in fractions 1 and 2, material from an equivalent of 4×10^8 cells was used in panels A and B for immunoprecipitation of these fractions. In panels C and D, immunoprecipitation was carried out with an equivalent of material from 10^8 cells in the case of fractions 1 and 2. Immunoprecipitation from all other fractions was from material equivalent to 4×10^9 cells. The positions of the bands corresponding to fully elongated precursor maltose-binding protein (pMBP), mature maltose-binding protein (mMBP), and nascent matured maltose-binding protein (nMBP) are indicated. The positions in the gradient corresponding to cytoplasmic membrane (determined by density and β-NADH oxidase activity) and to outer membrane (determined by density, the presence of the major outer membrane proteins, and the presence of KDO) are indicated by the open and solid arrows, respectively. (A) Distribution of wild-type maltose-binding protein in minimally perturbed cells. A culture of exponentially growing *E. coli* K-12 strain G6 producing wild-type maltose-binding protein was treated as described in the legend to Fig. 3. (B) Distribution of wild-type precursor accumulated by dissipation of proton motive force. See Materials and Methods for details. (C) Distribution of wild-type maltose-binding protein accumulated after inhibition of protein synthesis. See Materials and Methods for details. (D) Distribution of mutated precursor that carries a defective leader peptide. See Materials and Methods for details.

in association with membranes (Fig. 4A; flotation data not shown). However, the distribution of labeled maltose-binding protein became identical to that of the maltose-binding protein present at steady state (detected by staining with Coomassie blue) when an excess of nonradioactive methionine was added 15 s after the addition of the isotope and growth was continued for 10 min; greater than 95% of the matured maltose-binding protein became soluble. Thus, it seems that cleavage of the leader does not instantaneously release the mature protein from the membrane. This observation supports the proposal made by others (14, 19, 21) that during export of periplasmic proteins there is a separate and distinct release step.

Approximately 25 to 30% of the fully elongated wild-type precursor was present in the gradient distributed among fractions 1 through 3 (not visible in Fig. 4A because of the quantity of those fractions applied to the gel), and the remainder was found in fractions which also contained cytoplasmic membranes (Fig. 3 and 4; density, 1.16 g/ml). The membrane association of the precursor in these gradient fractions was confirmed by adding sufficient metrizamide to bring the density of the gradient fraction to 1.29 g/ml and subjecting that sample to analysis by flotation centrifugation as described above. Greater than 85% of the precursor floated to the membrane position. After sedimentation centrifugation, the discrete species of nascent polypeptides, which we showed were membrane associated by flotation centrifugation (Fig. 2), were distributed between two membrane fractions that differed in buoyant density (Fig. 4A). One fraction contained the fully elongated precursor and had a density characteristic of cytoplasmic membrane (1.16 g/ml); the other had a density of 1.21 g/ml, close to that of outer membrane (1.22 to 1.23 g/ml). This fraction contained almost half of the nascent polypeptide species but contained insignificant quantities of fully elongated precursor. When elongation of polypeptides was arrested by the addition of chloramphenicol, nascent maltose-binding protein accumulated in both membrane fractions (Fig. 4C). It has been shown previously (29) that the discrete species of nascent polypeptides seen in Fig. 4A are proteolytically matured. Since cleavage of the leader occurs on the periplasmic side of the membrane (10, 39) and since these polypeptides were in the process of being synthesized when the cellular processes were stopped, they must span the membrane. Thus, they can be considered markers for domains of the membrane that are actively engaged in translocation of polypeptides.

Polypeptides that had not yet been proteolytically matured, whether they were fully elongated or nascent, were found in the fractions with the density characteristic of cytoplasmic membrane and not in the denser fraction. Nascent polypeptides that carry the leader are not as easily detected as the matured nascent species shown in Fig. 4A. A discrete nascent precursor species does exist; however, it has the same molecular weight as the fully elongated matured maltose-binding protein and thus the two are not resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Therefore, to determine which membrane fractions contained nascent chains that had not been proteolytically processed, we used two-dimensional proteolytic peptide mapping (17) designed to detect nascent chains of molecular weight 36,000 or higher among the polypeptides that were immunoprecipitated from each fraction of a sedimentation gradient similar to that shown in Fig. 4. The great majority of nascent precursor was found in fractions 4, 5, and 6 (density, 1.11 to 1.18 g/ml), with very little detectable in other fractions. To account for the absence of polypeptides carrying the leader sequence in association with the dense membrane fraction, it seems reasonable to assume that translocation followed by proteolytic processing occurred immediately after precursor species had entered those domains. This assumption is supported by the observation that when the cells were incubated for 1 min with the uncoupler CCCP before they were labeled with [^{35}S]methionine, fully elongated, wild-type precursor accumulated in the dense fraction (Fig. 4B). In all cases, the

fractions isolated by sedimentation centrifugation were subsequently analyzed by flotation gradient centrifugation to confirm the conclusion that the polypeptides were membrane associated (data not shown).

Membrane association of a precursor carrying a mutationally altered leader with sites of initial interaction and with sites of translocation. Analysis of the membrane-association of precursor MalE18-1 by sedimentation gradient centrifugation showed that the mutated, intact precursor was found in the fractions corresponding to those containing wild-type precursor and that were operationally defined above as sites of initial interaction. In addition, intact mutant precursor was detected in the denser fractions (density, 1.21 g/ml) that have been operationally defined as translocation sites. Thus, it appears that the mutational change in the leader does not completely prevent initial membrane association or transfer to the translocation site. We conclude that the membrane-associated mutant precursor is defective in translocation and not a subsequent step, such as release from the periplasmic face of the membrane, since pulse-labeled precursor MalE18-1 was not accessible to proteinase K that was added to intact spheroplasts, but was completely degraded if the protease was added to spheroplasts that had been lysed (C. M. Gates and L. L. Randall, unpublished results). Further evidence that the mutant precursor remains on the cytosolic side of the membrane comes from the fact that the precursor present at steady state was recovered in a degraded form primarily from the cytosolic fraction (data not shown). The origin of the degraded material is unclear. The mutational change within the leader might affect the efficiency of entry into the pathway so that the precursor which was degraded might not have associated with the membrane, or the precursor might have bound to and subsequently been released from either the entry or the translocation site.

Conformational state of the precursor. Using sensitivity to degradation by proteinase K as an assay of conformational state, we previously established an inverse correlation between competence for export and attainment of stable tertiary structure (27). We demonstrated that precursor maltose-binding protein lost the ability to be exported when it had folded into a conformation similar to that of the mature protein, and we proposed that the competent state was maintained by interaction of the precursor with the export apparatus. Using the same assay of conformation, we now show that precursor species bound to the membrane are maintained in a state devoid of the stable structure of the mature species. Gradient fractions containing the precursor to be assessed were incubated with proteinase K as described in Materials and Methods. The fractions tested were those containing precursor associated with membrane of density 1.16 g/ml and those containing precursor bound to membrane of density 1.21 g/ml. Both the wild-type precursor and the mutated MalE18-1 precursor in these fractions were completely degraded. The precursors remained sensitive to digestion even if the fractions were incubated at 30°C for 20 min before addition of protease. To demonstrate that the precursors were maintained in this protease-sensitive state by interaction with cellular components, samples of the gradient fractions were made to be 2 M in guanidinium hydrochloride. The guanidinium hydrochloride was then diluted to 0.05 M, and the samples were incubated to allow the denatured precursors to fold before conformation was assessed by addition of proteinase K. Under these conditions, both species of precursor, wild-type and mutated, from all fractions tested folded into a stable conformation, i.e., the protease removed the leader peptide but in all cases greater than 60% of the material was recovered in a form that migrated at the position of mature protein during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Thus, we conclude that if the precursor is uncomplexed it is free to fold; however, folding into a stable state, which is resistant to degradation by protease, is completely blocked by association with the export apparatus.

DISCUSSION

To elucidate the role of the leader peptide, we analyzed the membrane association of several species of precursor maltose-binding protein. For the purpose of discussion, the export process is divided into the following stages: (i) entry into the pathway, which encompasses all events occurring in the cytosol up to and including initial association with the membrane; (ii) translocation, which includes passage of the precursor from the site of initial association on the membrane to the translocation site as well as transfer of the polypeptide through the membrane; (iii) proteolytic removal of the leader peptide by leader peptidase; and (iv) release, which includes folding of the matured polypeptide and dissociation from the membrane on the periplasmic side. The investigations described here indicate that the leader peptide plays a role at the translocation stage as well as at the entry stage. The notion that leader peptides function at more than one stage of export has been presented before (18, 32, 34, 36). Moreover, it has been proposed (18) that the parameters defining an active leader peptide may be governed by the specific function under examination. The mutated leader carried by the MalE18-1 precursor is active in mediating entry into the export pathway, since newly synthesized precursor MalE18-1 was found associated with membrane, whereas a polypeptide synthesized without a leader did not bind to the membrane. Rasmussen and Bassford (30) examined the effect of the mutational change in the leader of MalE18-1 on the intracellular distribution of polysomes synthesizing that polypeptide. They observed a decrease in the polysomes synthesizing MalE18-1 that were membrane bound relative to the membrane-bound polysomes that were elongating the wild-type species. These results are not in conflict with those reported here, since it may be that the mutational change decreases the efficiency of association with the membrane. Rasmussen and Bassford (30) examined the distribution of short nascent polypeptides, the synthesis of which had been initiated at the time of fractionation of the cellular components, but the elongation occurred in vitro. In contrast, our analysis includes only the fully elongated precursors and nascent species that were very near completion at the time of cellular fractionation.

A subfraction of membrane having a density of 1.21 g/ml has been operationally defined as a translocation site, since when an unperturbed cell producing wild-type maltose-binding protein was fractionated, only processed nascent chains were found associated with these membranes; but when translocation was blocked by dissipation of the proton motive force, precursor maltose-binding protein accumulated in this fraction. In cells producing precursor MalE18-1, the fully elongated form of this mutated precursor was found in association with both the light and the dense membrane fractions. We conclude that the mutated leader allows not only entry into the pathway, but also transfer from the site of initial interaction (within membrane domains having a density of 1.16 g/ml) to the translocation site. Since essentially no matured MalE18-1 protein is found in the periplasm and since the precursor accumulates in the cytosolic compart-

ment, the defect in export must occur at the translocation step. The mutational change in this precursor is the substitution of an arginyl residue for a methionyl residue at position 18 in the hydrophobic core of the leader sequence (5). Gierasch and co-workers (6) have proposed that hydrophobicity of the leader is important during direct interaction of the precursor with the phospholipid bilayer. The work presented here suggests that such hydrophobic interactions are crucial for translocation and that other parameters render the leader functional during the entry stage.

We have preliminary evidence (C. M. Gates and L. L. Randall, unpublished results) that another mutated precursor, MalE14-1, which like MalE18-1 has a charged aminoacyl residue substituted for a neutral residue within the hydrophobic core of the leader (Pro-14 is replaced by Glu), initially associates with the membrane but is defective in the translocation stage. It has been proposed (23) that the role of the leader during this entry stage is to modulate the folding pathway of the precursor to create or expose a structural element that is recognized by the export apparatus. It has been directly shown that both the wild-type leader (23) and the leader of MalE14-1 (G. Liu, T. B. Topping, W. H. Cover, and L. L. Randall, in press) slow folding of the precursor relative to that of the mature protein. If the retardation of folding is crucial to initial membrane association, one would expect the leader of MalE18-1 to retard folding. Studies to demonstrate this are in progress.

Precursor maltose-binding protein is competent for translocation only if it does not attain the tertiary structure of the mature species (27). The presence of the leader, although it retards folding, was shown to be insufficient for maintaining the precursor in this competent state. We suggested that the precursors are kept from assuming the thermodynamically favored mature conformation by interaction with components of the export apparatus. This contention is supported by data presented here showing that the precursor species, whether wild-type or mutant, were sensitive to degradation by added protease when they were associated with the membrane but became resistant to proteolytic degradation when the interactions were disrupted. The association of polypeptides with the membrane was shown to occur before synthesis was complete, as indicated by the observation that nascent polypeptides carrying the leader showed behavior similar to that of the fully elongated species in both fractionation techniques used. Thus, it seems likely that the cytosolic factors that have been demonstrated in vitro to enhance translocation into membrane vesicles (9, 35) or to modulate folding of precursors (8) interact with nascent polypeptides.

In addition to insights into the role of the leader peptide, information about the site of export within the cell envelope can be derived from the studies described here. Markers for domains of the membrane actively engaged in export are provided by nascent polypeptides that have been proteolytically matured and thus are in the process of being translocated. Since the cytoplasmic membrane must be traversed for a protein to enter the periplasm, it was not surprising that these matured, nascent species were found associated with membrane having the density characteristic of bulk cytoplasmic membrane. However, the presence of nascent polypeptides associated with membrane of higher density (1.21 g/ml) was unanticipated. It is possible that some domains of membrane involved in translocation are particularly rich in protein or that rRNA remained bound at some translocation sites even though EDTA was present during cellular lysis. Preliminary analyses with immunoelectron microscopy (M. E. Bayer, J. R. Thom, and L. L. Randall, unpublished observations) support an alternative explanation for the unusually high density of cytoplasmic membrane engaged in export: the export sites may be clustered around zones of adhesion between the cytoplasmic membrane and the outer membrane. Vesicles derived from these regions, where the two membranes are in close apposition, have been shown to have a density between the densities of cytoplasmic and outer membranes in *Salmonella anatum* (4). Whatever the explanation, it is clear that nascent maltose-binding protein undergoing export in unperturbed cells is found associated with a membrane fraction that has a density close to that characteristic of outer membrane. In addition, when export was blocked by dissipation of the proton motive force, fully elongated precursor accumulated in this dense membrane fraction. These observations underscore the need for caution when using density gradient centrifugation as the criterion for proper localization of proteins to the outer membrane. Unnatural proteins such as chimeras composed of the leader sequence of an outer membrane protein fused to a cytosolic protein might appear to be translocated to the outer membrane when in fact they are retained in the export apparatus.

ACKNOWLEDGMENTS

We thank P. J. Bassford, Jr., for providing strain MC4100 and its derivatives. We are grateful to John Puziss for constructing strain JP27, to Traci B. Topping and James Tsuruta for determination of the amino acid sequence of MalEΔ2-26, and to Simon J. S. Hardy for establishing the conditions for flotation centrifugation.

This research was supported by Public Health Service grant GM29798 to L.L.R. from the National Institutes of Health.

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